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Integrase-mediated recombination of the *bel-1* gene cassette encoding the extended-spectrum β-lactamase BEL-1

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Running title: Integrase-mediated *bel-1* mobilization

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Integrons are genetic elements that can acquire and rearrange gene cassettes. The *bla*$_{BEL-1}$ gene encodes an extended-spectrum β-lactamase, BEL-1, that is present at second position of the variable region of class 1 integrons identified in *Pseudomonas aeruginosa*. The mobility of the *bel-1* gene cassette was analyzed in physiological conditions and with the integrase gene being overexpressed. Cassette mobility in *Escherichia coli* was detected by excision/integration into the recipient integron In*3* on the conjugative plasmid R388, with the over-produced integrase. Despite several antibiotic pressures, the *bel-1* cassette remained at the second position in the integron, highlighting its stability in *P. aeruginosa*. Overexpression of the integrase gene in *E. coli* induced the *bel-1* cassette recombination. However, cassettes containing two genes (*bla*$_{BEL-1}$*/smr2* or *bla*$_{BEL-1}$*/aacA4*) were excised suggesting that the *bel-1* cassette *attC* site was defective. We showed that *bel-1* is a stable gene cassette under physiological growth conditions irrespective of the selective antibiotic pressure, that may be mobilized upon over-overexpression of the integrase gene.

INTRODUCTION

Class 1 integrons are genetic elements that can acquire and rearrange gene cassettes including genes carrying antibiotic/disinfectant resistance genes, therefore participating to the evolution toward multidrug resistance. Integrons are bracketed by two segments at their 5' (5'-CS) and 3' (3'-CS) ends. The 5'-CS includes *intI1*, a gene encoding a site-specific recombinase of the DNA integrase family, *attI* being the cassette integration site, and the promoter P_c (sometimes associated with a second promoter P_2) driving the expression of the cassettes (1-5). The 3'-CS includes disinfectant (*qacEΔ1*) and sulfonamide (*sul1*) resistance determinants, and an open reading frame of unknown function (ORF5) (1, 2, 6). Between those two conserved regions occurs the variable region which basically is a gene cassette array.

The gene cassettes are independent units each consisting of a gene bracketed by copies of a recombination site named *attC* (also named 59-be) (1, 2, 6). The *attI* and *attC* sites do not share the same architecture. The *attI* site is conserved among class 1 integrons and possesses four integrase binding sites. Two of those integrase-binding sites (L and R) are located at the core-recombination sites, and two are 5' to the core site, organized as Direct Repeats (DR1, DR2) (1, 7, 8). Even though *attC* sites are poorly conserved among gene cassettes, they share

an imperfect inverted repeated structure, comprising two pairs of inversely oriented integrase-binding domains separated by a spacer of 7-8 bp (9, 10). The boundaries of the *attC* site are defined by degenerate core sites separated by a central region that is highly variable in sequence and size (20-104 bp) (11). The only sequences that are fully conserved within *attC* sites are two triplets, 5'-AAC-3' and 5'-GTT-3' (11). Nevertheless, *attC* sites present a conserved palindromic organization, allowing the formation of a cruciform structure through the extrusion and self-pairing of both DNA strands (1, 10). When folded, single-stranded *attC* sites present an almost canonical core site consisting of L''-L' and R''-R' duplexes separated by a bulged region (12-14). Both *attC* and *attI1* sites are involved in site-specific recombination catalyzed by the integrase IntI1 (15-18). During recombination leading to cassette integration or excision, recombination crossover occurs on the bottow strand only, between the G and TT of the seven-base core site, GTTRRRY (1, 10). Recombination can also occur between two *attI* sites and between two *attC* sites, but the most efficient is between an *attI* site and an *attC* site (18, 19).

Most of the gene cassettes are promoterless, and thus their expression depends on promoters in the *intI* gene or in the *attI* site that are oriented toward the integration point, namely the P_c/P_2 promoters (1, 5, 20). The excision activity of an integrase is inversely

proportional to the promoter strength (5), and this inverse correlation is due to a phenomenon of transcriptional interference. P_{int} and P_c are embedded and oriented in outward orientations, and a strong transcription from P_c hinders transcription from P_{int}, inhibiting expression of the integrase gene (21).

Numerous antibiotic resistance genes are found on integrons. The bla_{BEL-1} and bla_{BEL-2} genes, encoding extended-spectrum β-lactamases (ESBLs), have been identified in numerous *Pseudomonas aeruginosa* isolates (22-24). The *bel-1*/*bel-2* cassettes are associated with *aacA4* and *aadA5* gene cassettes coding for an aminoglycoside-modifying enzyme and also with the *smr* cassette encoding resistance to antiseptics (Figure 1) (22, 23).

We investigated here the putative mobility of the *bel-1* gene cassette and the role of several non-related antibiotic molecules to putatively induce its mobility.

RESULTS

Role of antibiotic exposure in *bel-1* mobility within In*120*. After 10 days of *P. aeruginosa* culture with sub-inhibitory concentrations of antibiotics, only 2.1-kb PCR products could be obtained, consistent with the permanent presence of the *bel-1* cassette at the

second position of the class 1 integron (Figure 2). That gene cassette therefore remained at the second position of the integron over time, under all conditions and exposure to non-related antibiotic molecules. The changes of MIC values for aztreonam and piperacillin between Day 4 and Day 10 were probably due to changes in cell permeability or in upregulation of efflux pumps that is likely to occur in *P. aeruginosa* (Table S1) (25).

Influence of the environment of the *bel-1* gene cassette on its mobility. As described above, the *bel-1* gene cassette associated with a native integrase expression in the presence or absence of various antibiotics remained stable (Figure 2). Therefore, the effect of IntI1 integrase overproduction was investigated. The Caz^R-Rif^R cointegrates were obtained only in strains containing p112.Kan (IntI1 overexpression) indicating that they are the result of integrase-mediated recombination (Table 1). The only sites available for recombination with In*3* in *p.Bel.Smr* and *p.IntI1.Bel.Smr* was the *bel-1 attC* site. The cointegrates that resulted from R388::pBel conjugation were selected as Caz^R-Rif^R colonies, and were found at only a very low frequency (10^{-5}) (Table 1) suggesting that the *bel-1 attC* site was likely inefficient for recombination with the *attI1* site or an *attC* site in In*3*. The cointegration frequency was 5 to 10 fold higher for *pAac.Bel.Smr.Aad, p.Bel.Smr.Aad,* and *p.IntI1.Bel.Smr.Aad*, all carrying the *smr-2 attC* site, than for *p.Bel.Smr* and *p.IntI1.Bel.Smr*,

which carry a *bel-1 attC* site. The *smr2 attC* site was thus more efficient for recombination than the *bel-1 attC* site itself. All these constructs contained a highly efficient recombination site (*smr2 attC*) that was likely mainly involved in the cointegration process. Overall, the cointegration frequencies seemed to depend on the recombination efficiency of the *attC* site available for recombination in the donor integron.

DISCUSSION

Recombination assays were carried out to investigate the recombination activities of the sites surrounding the *bel-1* gene cassette encoding the ESBL BEL-1 in *P. aeruginosa*. *E. coli* DH10B, containing an inactivated form of the recombinase RecA (*recA1*) limiting recombination between homologous sequences was used for that purpose. This feature ensures that the recombination events involved IntI1-mediated site-specific recombination and not RecA-mediated homologous recombination. The excision/mobilization experiments showed that the *attC* site of the *bel-1* gene cassette was inefficient, and that this gene cassette was not mobilizable independently. This might likely been explained by the sequence of the *bel-1 attC* site itself, that does not correspond to the ones being better recognized by the Int1 integrase, as demonstrated previously (*attC* containing the T-N6-G or T-N6-C sequence) (26). Here the extrahelical bases constituting the *bel-1 attC* bottom strand (respect to Figure 3) are

distantly related from those being well recognized by Int1. We also showed that the *smr2 attC* is enhanced over the *bel-1 attC* as it is involved in almost all recombination events when both gene cassettes are present on a plasmid. *Smr2* always remained associated with the *bel-1* gene cassette. Noticeably, most of the gene cassettes were unnecessary for bacterial growth, and may be lost when the integrase is overexpressed in the absence of corresponding selection. The excision of gene cassettes, considered as independent units, depends on the flanking recombination sites, *attC* in this case.

A study revealed a similar phenomenon for the excision of the *veb-1* gene cassette (encoding another ESBL, namely VEB-1, also found in *P. aeruginosa*) that occurs at only low frequency due to an inefficient *attC* site (27). The *aadB* gene cassette includes a highly efficient *attC* site that allows efficient *veb1-aadB* coexcision (27). This coexcision might also explain the frequent association between the *veb-1* and *aadB* gene cassettes in those class 1 integrons found in clinical isolates. Here we observed the same phenomenon with the *bel-1* donor gene cassette tandem.

Overall, our work provides some insights into the organization of $bla_{\text{BEL-1}}$-containing integrons. It is likely that those latter evolved from a common ancestor carrying an early association between the *bel-1* and *smr2* gene cassettes (28). It is also possible that *smr2* was

responsible for *bel-1* gene cassette recruitment and of the co-mobilization of *bel-1-smr2* into class 1 integrons. Although bla_{BEL-1}-containing integrons are subject to gene cassette rearrangements, we propose that the nature of *bel-1 attC* stabilizes its genetic environment, probably by impairing recombination events that could lead to its loss.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions and MIC determination. The bla_{BEL-1}-positive *P. aeruginosa* clinical isolate 51170 was from a previous study (Table S2) (22). Its class 1 integron contained the *bel-1* gene cassette (Figure 1). The recombination-deficient strain *Escherichia coli* DH10B (Life Technologies, Eragny, France) was used for bacterial electroporation experiments and rifampin resistant *E. coli* DH10B-Rif for conjugation experiments. The self-conjugative plasmid R388 (TmpR, Tra+) carrying an integron (In*3*) that contains the *dfrB2* gene cassette encoding resistance to trimethoprim (Tmp) was used in our experiments as an integration-recipient plasmid (29). Plasmid p112 (a pTRC99A derivative), conferring resistance to kanamycin and containing the *intI1* gene under the control of an IPTG-inductible synthetic P_{trc} promoter, was used for experiments in conditions of integrase gene overexpression (30). The low-copy number vector, tetracycline resistant, pBBR1MCS.3, was used for cloning experiments (31). Bacterial cells were grown in

Trypticase Soy (TS) broth or on TS agar plates (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France) with the following antibiotics as appropriate; ceftazidime (15 µg/ml), rifampin (200 µg/ml), aztreonam (6 µg/ml), trimethoprim (25 µg/ml), tetracycline (15 µg/ml), kanamycin (30 µg/ml), gentamicin (12 µg/ml), piperacillin (12 µg/ml), and ciprofloxacin (8 µg/ml). MIC determinations were performed using disc diffusion and Etests (AB bioMérieux, La Balme-les-Grottes, France) by standard techniques according to the CLSI guidelines (32).

To investigate the stability of the *bel-1* gene cassette under antibiotic exposure, *P. aeruginosa* was grown 10 days with sub-inhibitory concentrations of gentamicin (12 µg/ml) to favorize the stabilization of the *aacA4* cassette (encoding reduced susceptibility to gentamicin) at the first position, of aztreonam (6 µg/ml), of piperacillin (12 µg/ml), and of ciprofloxacin (8 µg/ml) (likely to increase integrase expression by inducing SOS system). *P. aeruginosa* was also grown for 10 days without any antibiotic as a control. Aliquots were plated every day for bacterial counting and MIC determinations. Cassette mobility in *P. aeruginosa* was assessed daily for ten days. DNA was extracted from aliquots collected daily and INTIN and BELB primers were used in PCR experiments to detect cassette mobility (Table 2). In the absence of *bel-1* cassette mobilization, the expected size of the PCR

amplification product is 2.1 kb, although it is 1.5 kb if the cassette has been mobilized (Figure 2).

Nucleic acid extraction, PCR and sequencing. PCR experiments using FastStart Taq polymerase were performed using primers as listed in Table 1 and according to the manufacturer's recommendations (Roche Diagnostics, Meylan, France). The PCR products were purified over Qiaquick columns (Qiagen, Courtaboeuf, France). An ABI PRISM 3100 automated sequencer (Applied Biosystems, Les Ulis, France) and laboratory-designed primers were used for sequencing both strands. Recombinant plasmids were extracted using Plasmid Maxi Kits (Qiagen) according to the manufacturer's instructions. Total DNA was extracted as described (26).

DNA manipulations and plasmid constructions. T4 DNA ligase and restriction endonucleases were used according to the manufacturer's recommendations (Amersham Biosciences, Orsay, France). An HindIII-digested omega fragment (ΩKm) from plasmid pHP45Ω-Km (18), consisting of a kanamycin resistance gene flanked by transcriptional and translational termination sequences, was inserted into the HindIII site in the multiple cloning site of p112, resulting in recombinant plasmid p112.kan. The 1.2-kb EcoRI-BamHI fragment containing the *intI1* gene was excised from p112.Kan, then the ends were filled in by using

the Pfu DNA polymerase, and the plasmid was self-ligated, resulting in p112.KanΔ*int*. The primers listed in Table 2 were used to amplify fragments from genomic DNA of *P. aeruginosa* 51170, and the fragments inserted into *S*ma1-restricted pBBR1MCS.3 to give *pAac.Bel.Smr.Aad, p.Aac.Bel.Smr, p.Bel.Smr.Aad, p.Bel.Smr, p.IntI1.Bel.Smr, p.IntI1.Bel.Smr.Aad*, respectively (Figure 3). These plasmids were introduced into *E. coli* DH10B by electroporation as previously described (31). TS-agar plates containing tetracycline (15 µg/ml) and ceftazidime (15 µg/ml) were used for selection.

Induction of integrase expression. The various plasmids carrying the various amplified fragments were co-electroporated into *E. coli* DH10B (p112.Kan) or into *E. coli* DH10B (p112.KanΔ*int*). Strains were grown to stationary phase in TS-broth containing 15 µg/ml tetracycline and 30 µg/ml kanamycin, then diluted 1,000-fold into 200 ml TS-broth containing same antibiotics at same concentrations, and finally incubated until growth was exponential (OD_{600nm}, 0.5). The integrase gene expression was then induced by adding IPTG to a final concentration of 0.6 mM, and cells were harvested 2h later.

Excision / integration experiments. Integration of cassettes into the recipient integron In*3* on R388 was measured by mating-out assay experiments (Figure 4). Each of the following recombinant plasmids *pAac.Bel.Smr.Aad, p.Aac.Bel.Smr, p.Bel.Smr.Aad, p.Bel.Smr,*

p.IntI1.Bel.Smr, p.IntI1.Bel.Smr.Aad and pBBRMCS.3 (empty vector) were electroporated into *E. coli* DH10B (p112.kan; R388). Recombinant plasmid *pAac.Bel.Smr.Aad* was electroporated into *E. coli* DH10B (p112.KanΔ*int*; R388) as a negative control. Three single colonies were picked up for each condition and cultured overnight at 37°C in 10 ml TS broth containing 15 µg/ml tetracycline and 30 µg/ml kanamycin. These overnight cultures were diluted 10-fold in fresh TS without antibiotic and cultured under gentle agitation at 37°C for 1 h30. IPTG was then added (induction of *intI1* expression) and culture further continued for 2 h. Conjugation was performed by incubating 800 µl of recipient *E.coli* DH10B-Rif and 200 µl of the strain to be tested at 37°C for 3 h under gentle agitation. The mating mixture was then vortexed vigorously, placed on ice, and then plated. Aliquots of 100 µl of serial ten-fold dilutions were plated onto plates containing both trimethoprim (25 µg/ml) and rifampin (200 µg/ml), and both ceftazidime (15 µg/ml) and rifampin (200 µg/ml), respectively. For *E. coli* DH10B (R388; p112.kan; pBBRMCS.3), aliquots were plated either onto trimethoprim (25 µg/ml) plus rifampin (200 µg/ml), or tetracycline (15 µg/ml) plus rifampin (200 µg/ml) containing plates. The cointegration frequency was calculated by dividing the number of ceftazidime- and rifampin-resistant (Caz^R-Rif^R) transconjugants by the number of trimethoprim- and rifampin-resistant (Tmp^R-Rif^R) transconjugants.

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Figure legends

Figure 1. Schematic representation of integrons containing the bla_{BEL-1} gene found in clinical strain *P. aeruginosa* 51170 (22). Arrows indicate orientation of gene transcription. The *attC* and *attI1* sites are represented as black and grey circles respectively.

Figure 2. Schematic map of the plasmid constructs used in this study. Fragments were inserted into the multiple cloning site of the shuttle-vector, pBBR1MCS.3, represented as a solid line. The coding regions are shown as boxes with an arrow indicating the orientation of their transcription. The *attC* and *attI1* sites are represented as black and grey circles respectively. Incomplete *attI1* site are represented as truncated grey circles. The broken arrows indicate the P_c and P_{lac} promoters. (A) pAac.Bel.Smr.Aad; (B) p.Aac.Bel.Smr; (C) p.Bel.Smr.Aad (incomplete *attI1* site); (D) p.Bel.Smr (incomplete *attI1* site); (E) p.IntI1.Bel.Smr; (F) p.IntI1.Bel.Smr.Aad.

Figure 3. Schematic representation of a cassette excision/integration assay using *p.Bel.Smr.Aad* into the recipient integron In*3* on R388. The *attC* sites are represented as black circles. Incomplete *attI1* site are represented as truncated grey circles.

Figure 4. *bel-1* gene cassette mobility assay in conditions of native integrase expression before (D0) and after 4 (D4) and 10 days (D10) of antibiotic exposure. In the absence of cassette mobilization, the expected size of the PCR amplification product is 2.1 kb; the expected size is 1.5 kb when the cassette has been mobilized.

G: gentamicin; A: aztreonam; C: ciprofloxacin; P: piperacillin; T: negative control.

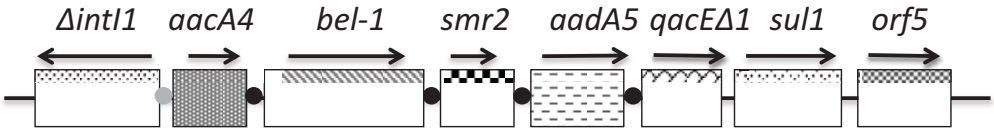
Table 1. Cointegration, cassette excision-integration frequencies.

Plasmid	**Sites**	**cointegration frequency** Mean ± SD
pAac.Bel.Smr.Aad	*attI1 ; aacA4-bel-1-smr2*	$6.8 .10^{-3}$ ($\pm$ $1.8.10^{-3}$)
p.Aac.Bel.Smr	*attI1 ; aacA4-bel-1*	$1.2. 10^{-3}$ ($\pm$ $4.35.10^{-4}$)
p.IntI1.Bel.Smr.Aad	*attI1 ; bel-1-smr2*	$5.33. 10^{-4}$ ($\pm$ $1.75.10^{-4}$)
p.IntI1.Bel.Smr.Aad	*attI1 ; bel-1*	$1. 10^{-4}$ ($\pm$ 9.10^{-5})
p.Bel.Smr.Aad	*attI1 ; bel-1-smr2*	$8.3.10^{-5}$ ($\pm$ $6.3.10^{-6}$)
p.Bel.Smr	*attI1 ; bel-1*	$1. 10^{-5}$ ($\pm$ $5.56.10^{-6}$)

Table 2. Sequences of primers used in this study

Name	Séquence 5'- 3'	Target	Genbank #	Position	Reference
BEL-A	CGACAATGCCGC AGCTAACC	*bla*$_{BEL-1}$	DQ089809	1374-1393	This work
BEL-B	CAGAAGCAATTA ATAACGCCC	*bla*$_{BEL-1}$	DQ089809	1822-1802	This work
INTIN	GCCAGGGCAGA TCCGTGCAC	*intI1*	AF133699	716-735	(30)
AttI1-belfor	GCCCTAAAACAA AGTTAGACGTAA GCCTATAATCTC	*bel-1*	DQ089809	306-318 958-980	This work
AttI1-belrev	TAGGCTTACGTC TAACTTTGTTTT AGGGCGACTGC	*attI1*	DQ089809	958-973 300-324	This work
Smr-rev	CGCGACCGCAAT GCCAACAC	*smr2*	DQ089809	2044-2063	This work
Aad-rev	CGAGCGTGGGA CAGCTGCTT	*aadA5*	DQ089809	2369-2388	This work
5'CS	GGCATCCAAGCA GCAAG	5'CS class 1 integron	DQ089809	204-220	This work
3'CS	AAGCAGACTTGA CCTGA	3'CS class 1 integron	AF133699	5381-5397	(30)

•**Figure 1**



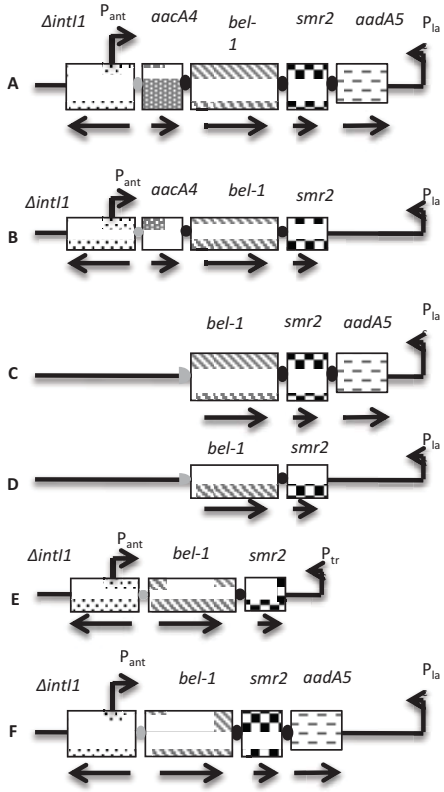


Figure 3

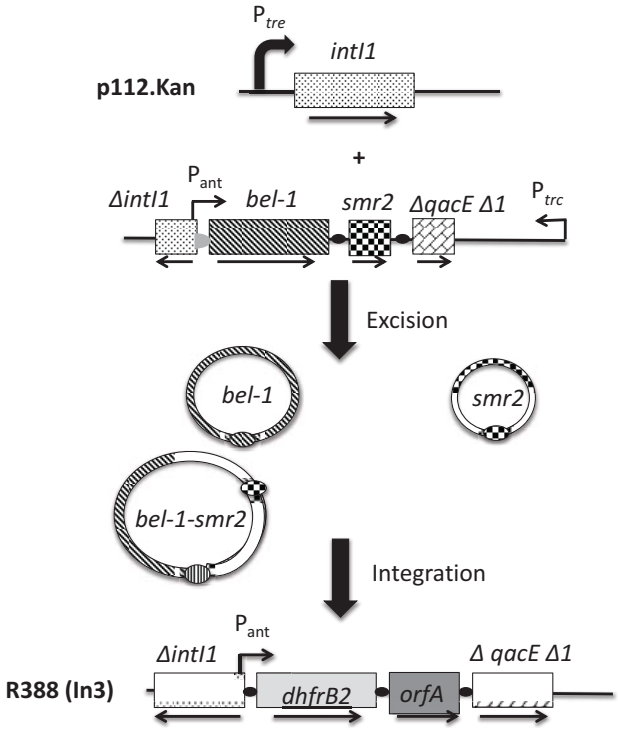


Figure 4